Mutational analysis of conserved AAA$^+$ residues in the archaeal Lon protease from *Thermoplasma acidophilum*

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Abstract The Lon protease from the archaeon *Thermoplasma acidophilum* (*Ta*Lon) is composed of an N-terminal ATPase associated with various cellular activities (AAA$^+$) domain and a C-terminal Lon protease domain. Although related in sequence to the soluble Lon proteases, *Ta*Lon was shown to be membrane-bound in its native host and also when expressed in *Escherichia coli*. Recombinant *Ta*Lon was purified as a functional high-molecular weight complex displaying ATPase and proteolytic activity. Mutagenesis of conserved AAA$^+$ residues revealed that the Walker A and B motifs, and the sensor 1 and sensor 2′ residues were essential for the ATPase activity, while the sensor 2 and the arginine finger were involved in activation of the protease domain.

Keywords: ATPase associated with various cellular activities; Archaea; Lon protease

1. Introduction

Lon is a one-component ATP-dependent protease, where the ATP-hydrolysing ATPase associated with various cellular activities (AAA$^+$) domain and the proteolytic domain are fused consecutively in a single polypeptide chain [1]. Lon is universally distributed and found in bacteria, eukaryotic organelles and archaea [2–6]. The best-studied representative is *Escherichia coli* Lon (*Ec*Lon), also called La, a non-essential heat-shock protein that degrades abnormal and short lived regulatory proteins [7,8]. Lon proteases from many other bacteria have been studied and confirmed the importance of Lon in regulated protein degradation [9].

Surprisingly, sequence analysis predicted two transmembrane regions for the *Thermoplasma* Lon homologue (*Ta*Lon) [10], while all bacterial and eukaryotic Lon polypeptides are devoid of transmembrane regions and are isolated as soluble proteases [7]. More recently, the Lon protease from *Thermococcus kodakarensis* (*Tk*Lon) was shown to be located in the membrane, but was purified from the soluble fraction when expressed in *E. coli* [11]. In contrast to bacteria, which have a multitude of energy-dependent proteases, archaea contain only two ATP-dependent proteases, namely the soluble proteasome–PAN system and the Lon protease [12,13]. Thus, archaea lack a membrane-bound protease, like the FtsH protease of the bacterial cytoplasm membrane and the m- and i-AAA proteases of the mitochondrial inner membrane [14–17]. Therefore, it is tempting to speculate that the archaeal Lon protease is a functional homologue of the bacterial and mitochondrial membrane-bound proteases and is responsible for the degradation of membrane proteins.

In this study, we showed that the *Ta*Lon protease is membrane-associated in *Thermoplasma* cells and when expressed in *E. coli*. The recombinant *Ta*Lon protease was purified from the membrane-fraction as a defined complex with ATPase activity. The proteolytic activity was processive, as described for related ATP-dependent enzymes [3]. A systematic mutational analysis of conserved AAA$^+$ residues yielded *Ta*Lon mutants impaired to different extents in ATPase and protease activity. Comparative analysis of wt and mutant *Ta*Lon revealed insights into the individual role of conserved AAA$^+$ residues in the intramolecular regulation of this ATP-dependent protease.

2. Materials and methods

2.1. Fractionation of *Thermoplasma acidophilum* cells

Prof. Dr. Karl O. Stetter (University of Regensburg, Germany) kindly provided *Thermoplasma* cells. Whole cell extracts were generated by thawing frozen *Thermoplasma* cells in Tris-buffer (50 mM Tris, pH 7.5, 5 mM $MgCl_2$, and 1 mM ATP) on ice. Under stirring, the pH of the cell suspension was adjusted to 7.0 with 1 M Tris, pH 11. After the addition of DNaseI, the cells were disrupted by two cycles at 15 000 psi using an EmulsiFlex-C5 (Avestin) cell disrupter. The suspension was centrifuged (15 min, 12 000×*g*, 4 °C) and the supernatant (whole cell extract (H)) was applied to an additional 200 000×*g* centrifugation step (2 h, 4 °C) separating cytosol from the membrane pellet.

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Abbreviations: AAA$^+$, ATPase associated with various cellular activities; AMC, 7-amido-4-methylcoumarin; AMPPNP, adenosine 5′-(β,γ-imino)-triphosphate; DDM, dodecyl-β-D-maltopyranoside; *Ec*FtsH, *Escherichia coli* FtsH; *Ec*Lon, *Escherichia coli* Lon; FITC, fluorescein isothiocyanate; IPTG, isopropyl-1-thio-β-D-galactopyranoside; MNA, 4-methoxy-2-naphthylamide; Suc, succinyl; *Ta*Lon, *Thermoplasma acidophilum* Lon; *Ta*PIP, *Thermoplasma acidophilum* proline iminopeptidase; *Tk*Lon *Thermococcus kodakarensis* Lon; wt, wild-type

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2.2. Cloning of the Thermoplasma lon gene

The full-length lon_{ta} was amplified from genomic DNA using the following primers: 5′-*ggaattccatatg*gaagaaaacattgagagcgtcgagg-3′ (sense, *Nde*I site underlined) and 5′-*ccgctcgagagctgcattatttcctg-ctcttgtgg*-3′ (antisense, *Bam*HI site underlined). The PCR product was digested with *Nde*I and *Xho*I and ligated with pET22b(+) (Ligation Kit, TakaraShuzo). The resulting plasmid (pET22b(+)-*Ta*Lon-His_6) encoded for full-length *Ta*Lon and two additional residues (leucine and glutamate) before the C-terminal His_6-tag. Eurogentec generated the anti-Lon peptide antibody. Anti-His_6 antibody was purchased from Santa Cruz Biotechnology.

2.3. Generation of active site mutants

Site-directed mutagenesis was performed using the QuickChange® Kit (Stratagene). pET22b(+)-*Ta*Lon-His_6 served as PCR template for the mutant proteins K63A, D241A, N293A, R305A, R375A, and R382A. The respective primers are listed with the mutated codons underlined: K63A 5′-ggagagccgggtactggagcctcgatgcttgcacagtcaatgg-3′ (sense), 5′-ccattgactgtgcaagcatc-gaggctccagtaccggctctcc-3′ (antisense); D241A 5′-gggtgtcctattcataggccgagataaaccttctcaggcccg-3′ (sense), 5′-cggg-gcctgagaaggtttatctcggctatgaataggacaccc-3′ (antisense); N293A 5′-ctcg-ttgctgccggagcctatgatgcgata-agaaaatatgc-3′ (sense), 5′-gcatatttcttatcgcat-cataggctccggcagcaacgag-3′ (antisense); R305A 5′-gcaccctgcg-ctcgcgt-caaggatacgcgg-3′ (sense), 5′-ccgcgtatccttgacgcgagcgcagggtgc-3′ (anti-sense); R375A 5′-gctgacct-tgaggctcgcggagctgggcggactcg-3′ (sense); 5′-cgagtccgcccagctccgcgagcctcaaggtcagc-3′ (antisense); R382A 5′-gctg-ggcggactcgtcgcggtagccggagatatagc-3′ (sense), 5′-gctatatctccggctaccgcg-acgagtccgcccagc-3′ (antisense). All plasmids were checked by DNA sequencing of the full-length gene (MWG).

2.4. Expression and purification of TaLon

Cells of *E. coli* BL21(DE3)C43 (Avidis) harbouring pET22b(+)-*Ta*LonHis_6(-wt, -K63A, -D241A, -N293A, -R305A, -R375A or -R382A) were grown at 37 °C in LB medium containing 100 μg ampicillin/ml. At an optical density of 0.6 (OD 600 nm), isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM. After 4 h the cells were harvested (4000×*g*, 10 min, 4 °C), washed with 50 mM Tris, pH 7.5, and stored until purification at −80 °C. For purification of *Ta* LonHis_6 (72 kDa), about 20 g of cells were thawed in buffer A (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 20 mM $MgCl_2$, and 10 mM imidazole) and disrupted in four cycles at 15 000 psi using an EmulsiFlex-C5 (Avestin) cell disrupter. The suspension was centrifuged (15 min, 12 000×*g*, 4 °C) and the supernatant was applied to a 200 000×*g* centrifugation step (2 h, 4 °C). The pellet containing the membrane fraction was diluted to a total volume of 100 ml in solubilisation buffer (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 20 mM $MgCl_2$, 10 mM imidazole, and 1.5% (w/v) dodecyl-β-D-maltopyranoside, Anatrace (DDM)). Solubilisation of the membranes was carried out for 2 h at 4 °C under gentle stirring and followed by centrifugation (1 h, 100 000×*g*, 4 °C). The remaining supernatant was applied to a 25 ml Ni^{2+}–NTA (Qiagen) column (0.5 ml/min, 4 °C). The column was washed with 10 column volumes (CVs) of buffer B (50 mM Na_2HPO_4, pH 8.0, 300 mM NaCl, 20 mM $MgCl_2$, 20 mM imidazole, and 1 mM DDM) and eluted with a linear gradient of imidazole (20–250 mM) in buffer B (10 CVs). The fractions containing *Ta*Lon were pooled, concentrated (Jumbosep 100K, PallFiltron), and applied to a Superdex 200 gel filtration column (HiLoad 26/60, Amersham Biosciences) equilibrated in buffer C (50 mM MES, pH 6.2, 300 mM NaCl, 20 mM $MgCl_2$, and 1 mM DDM). After separation (2 ml/min), the fractions containing *Ta*LonHis_6 were pooled, concentrated and stored at −80 °C.

2.5. Peptide hydrolysis

Peptidase activity of *Ta*Lon was assayed by hydrolysis of the fluorogenic peptide Suc-LLVY-AMC (succinyl-LLVY-7-amido-4-methylcoumarin, Bachem). The release of the fluorophore AMC was recorded over time (excitation at 320 nm, emission at 460 nm) with a fluorescence spectrophotometer (Fluostar Optima, BMG Labtechnologies). The assay was performed in a total volume of 200 μl in a 96-well plate. 4 μg *Ta*Lon (46.3 nM; assuming a hexameric complex) and 12.8 μg PIP (1.83 μM) [18] were incubated with 100 μM Suc-LLVY-AMC in assay buffer (50 mM MES, pH 6.2, 20 mM $MgCl_2$, and 0.5 mM DDM) with or without 2 mM of nucleotide (ATP, ADP, ATPγS or adenosine 5′-(β, γ-imino)-triphosphate (AMPPNP)) at 60 °C [18]. The degradation of Suc-LLVY-AMC by PIP alone was substracted and the initial slope of the curve was calculated.

2.6. Degradation of FITC casein

5 μg (58 nM, assuming a hexameric complex) *Ta*Lon was incubated with 25 μg (5 μM) fluorescein isothiocyanate (FITC) casein in 200 μl assay buffer with or without 2 mM nucleotide (ATP, ADP, ATPγS or AMPPNP). Degradation of FITC casein was monitored continuously (excitation at 480 nm, emission at 520 nm) in a fluorescence spectrophotometer (Fluostar Optima, BMG Labtechnologies) at 60 °C. The blank curve of FITC casein incubated in assay buffer was taken as a baseline and the initial slopes of reaction curves were calculated.

2.7. ATPase activity

ATPase activity was measured by determining the amount of inorganic phosphate formed upon ATP hydrolysis as a complex with malachite green and ammonium molybdate [19]. 25 μl of assay buffer containing 0.75 μg *Ta*Lon was preincubated for 10 min at 60 °C with or without 5 μM of FITC casein. The reaction was started by addition of 25 μl standard buffer containing 4 mM ATP. After further incubation for 20 min at 60 °C, the reaction was stopped by addition of 800 μl malachite green solution followed by 100 μl citrate solution (34% (w/v)). After 30 min incubation at RT, absorption at 640 nm was determined in a Perkin–Elmer UV/VIS spectrometer Lambda 40. The amount of phosphate in the sample was calculated from a standard curve of 0–15 nmol KH_2PO_4.

2.8. Reversed-phase HPLC analysis of degradation products

1.25 mg/ml β-casein (Sigma) was incubated with 20 μg/ml *Ta*Lon protease (46.5 nM; assuming a hexameric complex) in 600 μl of 25 mM MES, pH 6.2, with 10 mM $MgCl_2$, 1 mM DDM and 2 mM nucleotide at 60 °C. At different time points, aliquots of 40 μl were removed from the mixtures and 0.5 % TFA was added to stop the reaction before storage at −20 °C. 100 μl of each reaction mixture were analysed by reversed-phase HPLC. The column was equilibrated with 0.1% TFA in water and eluted with a linear gradient of 0–80% acetonitrile containing 0.08% TFA in 40 min at a flow rate of 0.3 ml/min and the gradient started 8.8 min after sample injection. Degradation products were detected by UV at 215 nm.

3. Results

3.1. TaLon is membrane-bound

During the sequencing of the *T. acidophilum* genome an open reading frame (ORF 1081) was identified, which showed significant sequence similarity to Lon proteases [10]. The *T. acidophilum* Lon (*Ta*Lon) protease encompasses an N-terminal AAA^+ domain and a C-terminal protease domain, but lacks the N-terminal α-helical domain inherent in most bacterial and eukaryotic Lon homologues. However, the AAA^+ domain of *Ta*Lon contains an insert between the Walker A and Walker B ATPase signatures [20] (Fig. 1), which is not present in any bacterial or eukaryotic Lon sequence. This insert is found in all archaeal Lon homologues and is predicted to contain two consecutive transmembrane helices, suggesting that archaeal Lon proteases are membrane associated [10].

To test for expression and subcellular localisation of the *Ta*Lon in *T. acidophilum* cells, an antibody was developed against the N- and C-terminal peptides of the *Ta*Lon protein. Cytosol and membrane of *Thermoplasma* cells were separated by ultra-centrifugation and analysed by Western blotting (Fig. 2A). The Lon protease was highly enriched in the membrane fraction, which verifies the predicted membrane insertion. Membrane localisation was also shown for the Lon homologue from the extreme thermophilic archaeon *Thermococcus kodakarensis* (*Tk*Lon) [11]. Taken together, this suggests that the predicted transmembrane region of archaeal Lon

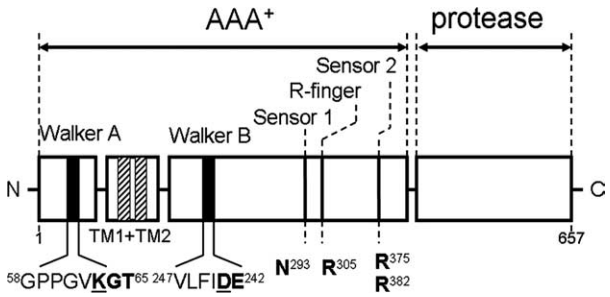

Fig. 1. Schematic representation of *Ta*Lon. *Ta*Lon is comprised of an N-terminal AAA^+ ATPase and a C-terminal Lon protease domain. The AAA^+ domain contains two transmembrane helices (TM1, TM2) inserted between the Walker A and B motifs. The positions of Sensor 1, Sensor 2′, Sensor 2 and the arginine finger (R-finger) are indicated with the respective amino acids.

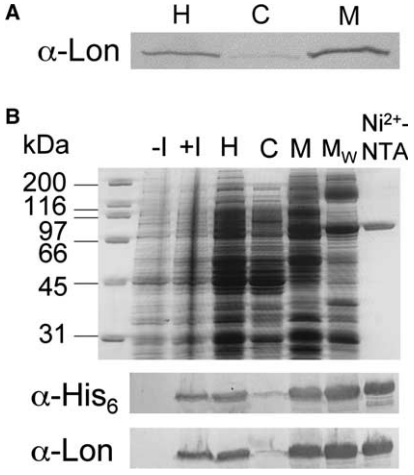

Fig. 2. Membrane localisation of native and recombinant *Ta*Lon protease. (A) *T. acidophilum* whole cell extract (H) was split by ultracentrifugation into cytosol (C) and membrane (M) fraction. The *Ta*Lon protease was detected by immunoblot analysis with anti-Lon antibodies (α-Lon). (B) Recombinant expression of $TaLonHis_6$ in *E. coli*. Samples from cell culture were taken before (−I) and after induction (+I) with 1 mM IPTG. The whole cell extract (H) was fractionated into cytosol (C) and membrane (M). To confirm the integral membrane character of $TaLonHis_6$, an aliquot of the membrane fraction was washed with 0.1 M Na_2CO_3, pH 11.5 (M_W). The crude membranes were solubilised and $TaLonHis_6$ was isolated by Ni^{2+}–NTA affinity chromatography. The top panel shows Coomassie-stained SDS-PAGE, the middle and lower panels represent the immunoblot analysis with anti-His_6 (α-His_6) and anti-Lon (α-Lon) antibodies, respectively.

proteases is responsible for membrane targeting. Homologues of the bacterial membrane-bound ATP-dependent protease FtsH have not been detected in the genome of any archaeon [12,13]. Thus, the archaeal membrane-bound Lon protease might represent a functional homologue of the bacterial FtsH protease and degrade archaeal membrane proteins.

3.2. Expression and purification of recombinant TaLon

The *T. acidophilum* lon gene was amplified from genomic DNA by PCR and cloned into the bacterial expression vector pET22b. Overexpression in *E. coli* led to membrane insertion of the *Ta*Lon protease (Fig. 2B), which indicated that the *Ta*Lon membrane anchor was functional in *E. coli*. The integral character of *Ta*Lon was confirmed by washing the isolated membranes with 0.1 M Na_2CO_3 (pH 11.5) (Fig. 2B). The isolated membranes were solubilised with detergent and recombinant *Ta*Lon was purified by Ni^{2+}–NTA affinity chromatography. Subsequent size exclusion chromatography revealed an apparent molecular weight of 430 kDa, which suggests that *Ta*Lon (72 kDa) assembles into a hexameric complex. Recently, the soluble protease domain from *Ec*Lon [21] and the AAA-domain of FtsH [22] were crystallised as hexameric complexes, supporting the hexameric model for *Ta*Lon. Preliminary electron microscopic analysis of *Ta*Lon revealed ring-shaped particles of unknown symmetry (data not shown).

3.3. Peptidase activity

In order to find the optimal conditions for the ensuing enzymatic characterisation, the ATPase activity of the recombinant enzyme was assayed at different temperatures, pH values and Mg^{2+} concentrations. Highest activity for *Ta*Lon was found at 75 °C, pH 5.75, and 10 mM $MgCl_2$. All subsequent assays, if not indicated differently, were done using these conditions except for the temperature, which was lowered to 60 °C in order to match the optimal growth temperature of *T. acidophilum* [23] and to reduce thermal autolysis of ATP.

To measure the peptidase activity of *Ta*Lon, a set of 18 diverse fluorogenic peptide substrates was tested. An increase in fluorescence was detected only for Suc-LLVY-AMC, and only after adding the *T. acidophilum* proline iminopeptidase (*Ta*PIP) to the reaction mixture. *Ta*PIP is a broad-spectrum aminopeptidase, which was shown to cleave preferentially after small and hydrophobic residues but does not cleave Suc-LLVY-AMC, due to the protecting N-terminal succinyl group [18]. In the absence of *Ta*PIP, the *Ta*Lon protease cleaved the Suc-LLVY-AMC peptide at the L–L and the L–V bonds as revealed by mass spectrometry, but did not release the fluorophore AMC (data not shown). In the presence of *Ta*PIP the products of the *Ta*Lon cleavages, LVY-AMC and VY-AMC, were further degraded and AMC was released as measured by increase in fluorescence.

The peptidase activity of *Ta*Lon is modulated by the ATP-state of its AAA^+ domain. In the absence of nucleotide, *Ta*Lon shows a basal activity that is slightly reduced in the presence of ATP or the non-hydrolysable analogues AMPPNP and ATPγS, whereas ADP exerts an inhibitory effect on the peptidase activity (Table 1). Modulation of the peptidase activity by nucleotides was described for *Ec*Lon, which is highly stimulated in the presence of ATP (10–30 fold) and strictly inhibited in the presence of ADP [7]. The inhibition of peptidase activity with increasing ATP concentrations described for *Tk*Lon [11] was neither observed for *Ta*Lon (data not shown) nor for *Ec*Lon [7].

3.4. Degradation of FITC casein

Protein degradation was monitored with FITC-labelled casein, a loosely folded protein [24] that is commonly used as a substrate for ATP-dependent proteases [7]. The fluorescence of the covalently linked FITC molecules is quenched, and only upon casein degradation and release of small peptide fragments carrying a FITC label the fluorescence increases [25,26].

Table 1
Relative ATPase and peptidase activities of wt and mutant *Ta*Lon[a]

		ATPase (%)	Peptidase (%)				
			No nucl.	ADP	AMPPNP	ATPγS	ATP
Wt		100 ± 4	100 ± 6	41 ± 3	93 ± 8	73 ± 4	89 ± 2
Mutant	Motif						
K63A	Walker A	0	113 ± 6	130 ± 5	138 ± 13	95 ± 13	103 ± 15
D241A	Walker B	5 ± 1	99 ± 5	58 ± 6	66 ± 5	58 ± 5	57 ± 7
N293A	Sensor 1	9 ± 1	122 ± 5	77 ± 6	103 ± 10	66 ± 12	78 ± 13
R305A	R-finger	38 ± 2	2 ± 1	3 ± 1	32 ± 11	81 ± 9	81 ± 7
R375A	Sensor 2′	2 ± 1	112 ± 5	56 ± 4	96 ± 5	74 ± 7	86 ± 8
R382A	Sensor 2	24 ± 3	6 ± 1	3 ± 1	28 ± 7	88 ± 6	86 ± 7

[a] ATPase and peptidase activities are given relative to the respective activities of the wt *Ta*Lon protease.

FITC casein degradation by *Ta*Lon is low in the absence of nucleotide, stimulated tenfold in the presence of ATP, and completely inhibited by ADP (Fig. 3A). In contrast to peptide hydrolysis (Table 1), AMPPNP and ATPγS do not efficiently promote casein degradation (Fig. 3A). The non-hydrolysable ATP analogues stimulate the reaction merely 1.5–2 fold, suggesting that translocation of larger substrates to the proteolytic sites is energy dependent (Fig. 3A and Table 2). Degradation of unfolded protein substrates by *Ec*Lon also requires ATP and is inhibited by ADP. Non-hydrolysable analogues of ATP allow degradation of casein by *Ec*Lon with ~20% of the rate observed with ATP, which perfectly matches with our data set [7].

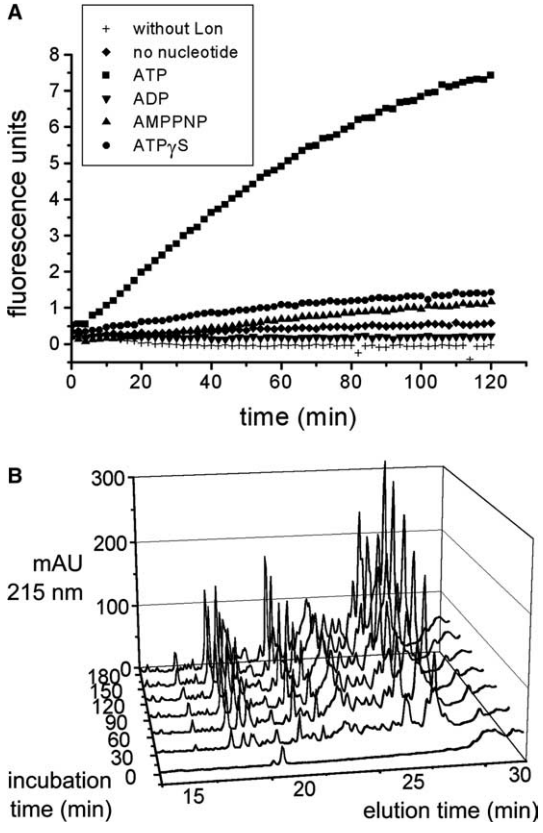

Fig. 3. Degradation of FITC casein. (A) 116 nM *Ta*Lon was incubated with 20 μM of FITC casein in assay buffer and fluorescence was measured over time in the absence or presence of different nucleotides (2 mM of ATP, ADP, ATPγS or AMPPNP). (B) Time course of β-casein degradation by *Ta*Lon. 46.5 nM *Ta*Lon was incubated with 50 μM β-casein in the presence of 2 mM ATP for 3 h at 60 °C. Every 30 min, a sample was taken from the reaction mixture and analysed by reversed phase chromatography. The degradation products were detected by absorption at 215 nm.

Time resolved analysis of β-casein degradation revealed processive proteolytic activity of *Ta*Lon (compare Fig. 3B). The elution patterns for all time points shown are identical, only the amount of the individual degradation products increased over the reaction time. This showed that *Ta*Lon degraded substrates in a processive manner, which is a characteristic feature of ATP-dependent proteases, e.g., *E. coli* ClpP protease, archaeal and mammalian 20S proteasomes, mammalian 26S proteasomes and *E. coli* and *T. thermophilus* FtsH proteases [26–30].

3.5. The AAA^+ mutants

In order to determine the function of conserved AAA+ residues of the *Ta*Lon, we generated a set of mutants including Walker A lysine (K63A), Walker B aspartate (D241A), sensor 1 asparagine (N293A), arginine finger (R305A), sensor 2 arginine (R382A) and an additional arginine residue in close proximity to sensor 2, dubbed sensor 2′ (R375A), the latter being absolutely conserved among archaeal Lon proteases (Fig. 1). Like *Ta*Lonwt all mutant proteins purified in this study assembled into high molecular weight complexes, as shown upon gel filtration (data not shown), indicating that the mutations did not affect oligomerisation.

The Walker A mutant *Ta*LonK63A lacked ATPase activity completely. As found for *Ta*Lonwt, the peptidase activity remained largely unaffected by addition of ATP or its non-hydrolysable analogues, but in contrast to *Ta*Lonwt the peptidase activity was not inhibited by ADP (Table 1). The protease activity of *Ta*LonK63A corresponded to the activity of *Ta*Lonwt in the absence of nucleotide or in the presence of non-hydrolysable analogues (Table 2). Importantly, this activity was not inhibited by ADP nor stimulated by ATP. Since the Walker A residue is responsible for coordination of the β- and γ-phosphate residues of ATP [20,31], *Ta*LonK63A is most likely impaired in nucleotide binding, explaining the indifference of its peptidase and protease activities against the addition of different nucleotides.

The Walker B mutant *Ta*LonD241A retained 5% ATPase activity. As the Walker B residue is involved in coordination of the β- and γ-phosphates via an Mg^{2+} ion [32], the greatly reduced ATPase activity of *Ta*LonD241A is most likely a consequence of impaired coordination. Remarkably, in the

Table 2
Relative ATPase and protease activities of wt and mutant *Ta*Lon[a]

		ATPase (%)[a]	FITC casein degradation (%)				
			No nucl.	ADP	AMPPNP	ATPγS	ATP
Wt		100 ± 2	9 ± 1	2 ± 1	15 ± 2	19 ± 1	100 ± 3
Mutant	Motif						
K63A	Walker A	0	12 ± 1	10 ± 1	12 ± 1	12 ± 1	19 ± 1
D241A	Walker B	8 ± 1	17 ± 1	9 ± 1	7 ± 1	13 ± 2	17 ± 3
N293A	Sensor 1	8 ± 1	10 ± 1	2 ± 1	11 ± 0	20 ± 2	25 ± 2
R305A	R-finger	43 ± 1	0	0	0	6 ± 2	65 ± 6
R375A	Sensor 2′	2 ± 1	12 ± 1	1 ± 1	8 ± 2	6 ± 1	9 ± 2
R382A	Sensor 2	53 ± 3	1 ± 1	0	1 ± 1	6 ± 1	69 ± 4

[a] ATPase activities were measured in the presence of 5 μM FITC casein. ATPase activities and casein degradation are given relative to the respective activities of the wt *Ta*Lon protease.

absence of nucleotide peptidase activity was indistinguishable from *Ta*Lonwt, but the peptidase activity was reduced to about 60% irrespective of whether ADP, ATP or its analogues were added (Table 1). This indicates that the binding of nucleotide without γ-phosphate (ADP) or with uncoordinated γ-phosphate inhibits the peptidase activity of *Ta*LonD241A. The degree of inhibition is comparable with the effect of ADP on *Ta*Lonwt. As a consequence of the lack of ATPase acitvity, the proteolytic acitvity was greatly impaired and could not be stimulated by addition of nucleotides (Table 2).

Sensor 1 is a conserved polar residue among AAA^+ proteins, which was proposed originally to sense the nucleotide state of the ATP-binding pocket [33,34]. However, meanwhile it was shown that mutation of the sensor 1 residue impairs the ATPase activity dramatically, suggesting that sensor 1 has a catalytic role [35,36]. Consistently, the sensor 1 mutant *Ta*LonN293A displayed very low ATPase activity, but the regulation of the peptidase activity by binding of different nucleotides remained largely unaffected (Table 1). The proteolytic activity was severely reduced, irrespective of the absence or presence of nucleotides (Table 2). Thus, we suggest a catalytic role for the *Ta*Lon sensor 1 residue in ATP hydrolysis.

Unexpectedly, mutating the sensor 2′ arginine, which is in close proximity to the sensor 2 residue and absolutely conserved in archaeal Lon proteases, reduced ATP hydrolysis of *Ta*LonR375A close to zero (Table 1). Notably, there is also a conserved arginine residue (R537) in the bacterial Lon proteases close to the sensor 2 residue (R542). In the recently published structure of the AAA^+ α domain of the *Ec*Lon protease, arginine 537 is located at the end of helix 2 followed by a short loop and sensor 2 arginine 542 at the beginning of helix 3 [37]. Since sensor 2′ points into the same direction as sensor 2, it might also hydrolyse and/or sense the γ-phosphate of a bound nucleotide, like described for sensor 2 in different proteins [38]. The peptidase activity was similar to *Ta*Lonwt and was inhibited by ADP (Table 1). The protease activity was severely reduced under all assay conditions (Table 2) resembling the sensor 1 mutant *Ta*Lon N293A.

Hexameric AAA^+ structures revealed that the arginine finger protrudes into the catalytic site of a neighbouring subunit pointing towards the γ-phosphate in a way, which would enable it to stimulate hydrolysis [33,39,40]. Similarly, sensor 2, located in the helical C-terminal subdomain of the AAA^+ domain, was shown to be involved in ATP hydrolysis or sensing of the nucleotide state, however not in a neighbouring but in its inherent subunit [34,38,41].

Mutation of the arginine finger or sensor 2 arginine to alanine resulted in a significantly decreased ATPase activity of the respective mutants *Ta*LonR305A and *Ta*Lon382A. More dramatically, the peptidase activity of both mutants was totally impaired in the absence of nucleotide or in the presence of ADP (Table 1). Addition of ATP or ATPγS stimulated the peptidase activity close to wt level, whereas AMPPNP for reasons not known stimulated only moderately (Table 1). We conclude that in the absence of a γ-phosphate both mutants adopted a conformation incompetent for peptide hydrolysis, which was tranformed into an active conformation when nucleotides containing a γ-phosphate were added. The proteolytic activity of both mutant *Ta*Lon proteases was extinguished completely in the absence of nucleotide, or in the presence of ADP or AMPPNP. Addition of ATPγS stimulated marginally, but ATP activated proteolysis up to 70% of wt activity (Table 2). Thus, the arginine finger and the sensor 2 arginine contribute to ATP binding and/or hydrolysis, but are essential for coupling of nucleotide hydrolysis to protein degradation.

4. Discussion

In this paper, we present a thorough characterisation of the archaeal Lon protease from *T. acidophilum*. *Ta*Lon was found to be membrane bound in *Thermoplasma* and when overexpressed in *E. coli*. It formed a stable homooligomeric complex of probably six subunits and no nucleotide was needed for assembly. *Ta*Lon showed ATPase and peptidase activity and degraded protein substrates processively, a common feature of ATP-dependent proteases. Peptidase activity of TaLon was only observed in the presence of an aminopeptidase. Interestingly, the membrane-bound, Zn^{2+}-dependent AAA-protease FtsH from *E. coli* (*Ec*FtsH) degraded the Suc-LLVY-AMC peptide in a similar way, i.e., release of fluorescence was only detected in the presence of an aminopeptidase [42]. The internal cleavage of the Suc-LLVY-AMC peptide and the inability to hydrolyse other peptides imply that *Ta*Lon and *Escherichia coli* FtsH (*Ec*FtsH) have rather narrow substrate specificity and might be unable to cleave the peptide-AMC bond. *Tk*Lon showed very low activity towards the peptides glutaryl-AAF-4-methoxy-2-naphthylamide (MNA) and Suc-FLF-MNA, which was inhibited by ATP. Unfortunately, peptidase activity of *Tk*Lon was not tested in the presence of an aminopeptidase [11].

For a more detailed analysis of the regulatory mechanisms within the *Ta*Lon oligomer, several ATPase mutants were generated and analysed with respect to their specific activities. We showed that among the highly conserved AAA^+ residues, lysine in Walker A, aspartate in Walker B, the sensor 1 asparagine and sensor 2′ arginine were essential for ATPase activity with only minor influence on the peptidase activity. In contrast, two highly conserved arginine residues, sensor 2 and arginine finger, contributed only to a minor extent to the overall ATPase activity. Instead, mutation of the respective residues to alanine turns *Ta*Lon from a nucleotide independent to a strictly nucleotide dependent peptidase. Interestingly, the presence of γ-phosphate restored peptidase activity of the sensor 2 and arginine finger mutants. The proteolytic acitvity, however, was only reconstituted in the presence of ATP. Therefore, we conclude that the γ-phosphate of ATP along with the conserved sensor 2 and arginine finger residues provided is essential for nucleotide-dependent activation of the protease domain. While sensor 2 was found to be involved in interdomain communication rather than ATP hydrolysis in Hsp104 [43], such a role is unprecedented for the arginine finger [38].

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